

ATP- and ADP-DnaA protein, a molecular switch in gene regulation

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DnaA protein functions by binding to asymmetric 9mer DNA sites, the DnaA boxes. ATP-DnaA and ADP-DnaA bind to 9mer DnaA boxes with equal affinity, but only ATP-DnaA protein binds in addition to an as yet unknown 6mer site, the ATP-DnaA box AGATCT, or a close match to it. ATP-DnaA protein binding to ATP-DnaA boxes is restricted to sites located in close proximity to DnaA boxes, suggesting that protein-protein interaction is required for its stabilization. We show that ATP-DnaA represses *dnaA* transcription much more efficiently than ADP-DnaA. DnaA is thus a regulatory molecule that, depending on the adenosine nucleotide bound, can bind to different sequences and thereby fulfill distinct functions.

Keywords: autoregulation of *dnaA*/BIAcore/initiation of replication/*oriC*/repression

Introduction

DnaA protein is essential for the initiation of chromosomal DNA replication in *Escherichia coli* and other bacteria. It binds specifically to an asymmetric 9 bp consensus sequence TT^A_TTNCACA, the DnaA box, present five times in the replication origin of *E.coli*, *oriC*. DnaA protein forms a specialized nucleoprotein structure at the origin containing 20–30 DnaA monomers. This results in unwinding of an AT-rich region adjacent to the DnaA boxes. The unwound region represents the entry site for the DnaB–DnaC helicase complex, followed by other proteins required to form the replication fork (Kornberg and Baker, 1992; Skarstad and Boye, 1994; Messer and Weigel, 1996; Kaguni, 1997).

DnaA protein has a high affinity for ATP and ADP (K_D of 0.03 and 0.1 μ M) (Sekimizu *et al.*, 1987). Both forms bind to DNA in a similar fashion but only ATP-DnaA protein, not the ADP form, is active in the unwinding reaction *in vitro*. The rate of exchange of bound ADP by ATP is slow, 50% in 40 min, but it can be stimulated by acidic phospholipids (Castuma *et al.*, 1993). An intrinsic low-level ATPase is essential for DnaA function (Mizushima *et al.*, 1997). It has been shown that loading of the β subunit of the DNA polymerase III at the end of the initiation process and subsequent replication stimulates the intrinsic ATPase activity and inactivates DnaA due to hydrolysis of bound ATP (Katayama *et al.*,

1998). Adenosine nucleotides thus have a regulatory role in DnaA protein function, but the mechanism is not understood.

Besides its functions in the initiation of replication, DnaA protein acts as a transcription factor for a number of genes. Binding of DnaA to DnaA boxes in promoter regions leads to repression of transcription, as found for the *dnaA* gene itself, the *mioC* gene, the *uvrB* gene and the *rpoH* gene. Activation was reported for the *glpD* gene and the *nrd* operon (for a review, see Messer and Weigel, 1997).

The *E.coli dnaA* transcription unit contains two promoters, *dnaAp1* and *dnaAp2*, with a DnaA box located between them. *dnaAp2* is the stronger promoter, it contributes 60–80% of the *dnaA* transcripts (Kücherer *et al.*, 1986; Polaczek and Wright, 1990). Transcription from both promoters is reduced upon overproduction of DnaA protein, whereas inactivation of DnaA in temperature-sensitive *dnaA* mutants leads to derepression of the *dnaA* gene (Atlung *et al.*, 1985; Braun *et al.*, 1985; Kücherer *et al.*, 1986). These experiments proved the concept of autoregulation for the *dnaA* gene. It was assumed that the appropriately located DnaA box between the promoters would mediate autoregulation. However, mutating the DnaA box did not lead to the expected derepression of the *dnaA* gene *in vivo*. Also, overproduction of DnaA protein in that mutant still caused a reduction of *dnaA* transcription (Smith *et al.*, 1997). It was suggested therefore that autoregulation of *dnaA* may be indirect.

In this paper, we analyze the differences between ATP- and ADP-DnaA proteins with regard to their ability to bind to the *dnaA* promoter region. We investigate protein-DNA interactions and cooperative binding of the two forms. We show that ATP-DnaA protein binds cooperatively to a new DNA consensus sequence: the ATP-DnaA box. In contrast, ADP-DnaA protein binds to 9mer DnaA boxes only. These results suggest a novel mechanism for *dnaA* autoregulation, and we postulate the existence of a molecular switch for alternative DnaA functions.

Results

ATP- and ADP-DnaA bind to different binding sites

Differential binding of ATP- and ADP-DnaA to the promoter region of the *dnaA* gene was analyzed by DNase I footprinting using a PCR fragment from the *dnaA* promoter region. This fragment contains the *dnaA* promoters *dnaAp1* and *dnaAp2*. Previously, the ATP and ADP forms of the DnaA protein were not studied individually with regard to their effect on *dnaA* regulation; rather, a mixture was used or ATP was added. The intrinsic ATPase activity of DnaA hydrolyzes some of the bound

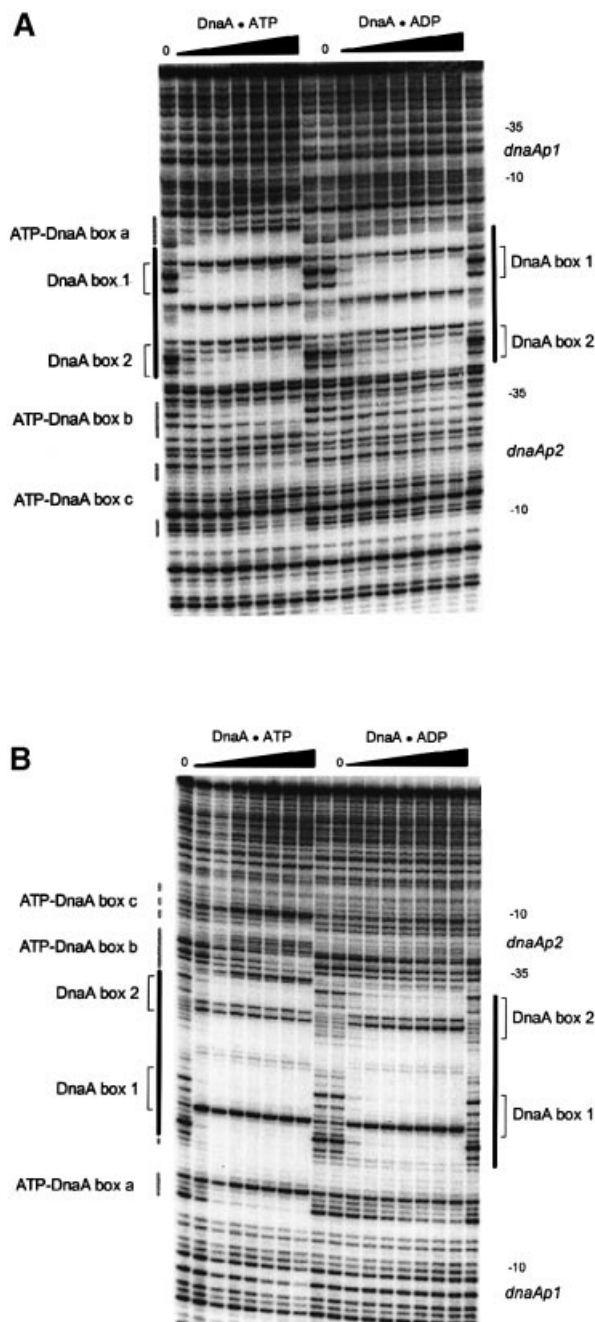


Fig. 1. ATP- and ADP-DnaA proteins show different protection patterns at the *dnaA* promoter. Footprint of (A) the lower strand and (B) the upper strand. DNase I footprinting was performed using a 198 bp PCR DNA fragment that was 5' end-labeled as described in Materials and methods. Increasing amounts of ATP- or ADP-DnaA were added, giving final concentrations of 12, 40, 72, 100, 168, 233 and 300 nM. Incubation was for 10 min at 37°C.

ATP, the ATP-ADP exchange rate is slow and ADP-DnaA represents the larger fraction of cellular DnaA (Katayama *et al.*, 1998). In order to study DNA binding with a uniform DnaA preparation, we therefore dialyzed the protein extensively against ADP or ATP (see Materials and methods). Figure 1 shows the results of a DNase I protection experiment with increasing DnaA concentrations using a 198 bp PCR fragment labeled at either 5' end. The highest DnaA concentration used, 300 nM, corresponds approximately to the *in vivo* DnaA concentra-

tion (Hansen *et al.*, 1991a). For ATP- and ADP-DnaA protein, we found binding to two regions for both strands: (i) to DnaA box 1, which conforms with the consensus sequence TT^A/TNCACA (Schaper and Messer, 1995); and (ii) to another site that has a mismatch from the consensus DnaA binding sequence and is located 12 bp downstream of DnaA box 1. This second site we call DnaA box 2. It has not been observed so far. ADP- and ATP-DnaA proteins bound to DnaA box 1 at identical low protein concentrations (12 nM). At slightly higher concentrations (40 nM), we found protection of DnaA box 2 in the case of ATP-DnaA protein; ADP-DnaA protein bound less well to DnaA box 2 (70–100 nM). For ATP-DnaA protein, we found three protected regions on both strands in addition to DnaA boxes 1 and 2. These regions share a consensus sequence; therefore we call them ATP-DnaA box a, b and c, respectively. We deduce from the protected DNA sequence that ATP-DnaA boxes have the consensus sequence AGATCT or a close match to it, which is different from the DnaA box consensus sequence. ATP-DnaA box a was bound at very low DnaA concentrations; complete protection was found in the range of 40–72 nM, comparable to DnaA box 2. ATP-DnaA boxes b and c were bound at higher concentrations, ~166 nM.

It has been found that one DnaA monomer bound to a DnaA box induces a bend of $42 \pm 4^\circ$ (Schaper and Messer, 1995). This bend causes distortions in the DNA, which result in DNase I-hypersensitive sites. ATP- and ADP-DnaA protein bound to the *dnaA* promoter induced different patterns of hypersensitive sites. ATP-DnaA protein caused several additional hypersensitive sites in the promoter region. This underlines the differences in complex formation of ATP- and ADP-DnaA protein. Details of DnaA boxes, ATP-DnaA boxes, protected and hypersensitive regions are summarized in Figure 2.

Kinetics and stoichiometry of DnaA-dnaAp complexes

For a more detailed analysis of complex formation at the *dnaA* promoter, we analyzed the kinetics and stoichiometry of the complexes observed in the DNase I footprints using surface plasmon resonance with the BIAcore instrument. Surface plasmon resonance measures the change in mass at the surface of a chip. Single DnaA boxes or different combinations of binding sites on biotin-labeled oligonucleotides were bound to a streptavidin-coated chip. A flow of ATP- or ADP-DnaA was applied past the chip. The major advantage of this technique is that protein-DNA interactions can be monitored in real time, very accurately and very sensitively. A response of 1 RU (resonance unit) corresponds to 1 pg/mm² protein. For DNA, we determined a correlation of 1 RU = 0.73 pg/mm² DNA (see Materials and methods). Since the BIAcore measures mass differences, one can calculate the mean stoichiometry of the complexes at a certain DnaA protein concentration. Using the BIAcore, one also has to consider certain limitations: (i) kinetics could only be determined for 1:1 interactions, using the Langmuir binding model, since correct assignment of a binding model is too error prone for more complex reactions; and (ii) *E. coli* DnaA tends to bind non-specifically to DNA and to the carboxymethyl dextran matrix of the sensor chips, since it is a very



Fig. 2. *dnaA* promoter region with protected and enhanced regions in DNase I footprints. Numbering corresponds to Hansen *et al.* (1982). DnaA boxes 1 and 2 are indicated by light gray arrows, protected regions by bars and hypersensitive sites by dots. Regions that show the same footprint pattern for both DnaA forms are marked in black. Regions with altered protection pattern or hypersensitive sites only seen with ATP-DnaA are shown in gray. ATP-DnaA boxes are in bold.

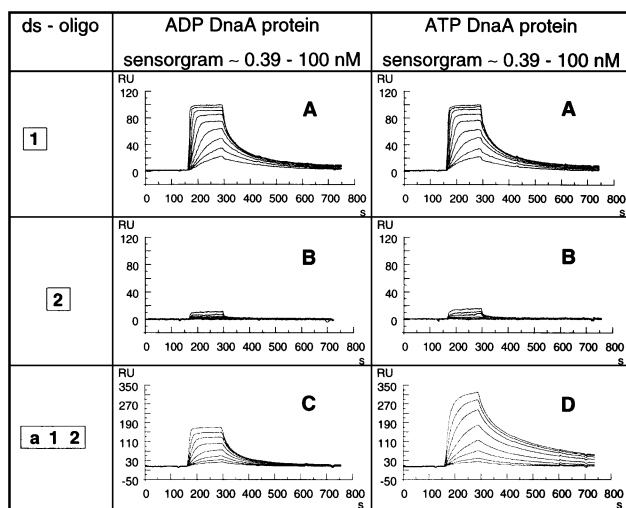


Fig. 3. Typical binding reactions of ATP- and ADP-DnaA proteins as measured by surface plasmon resonance. The different binding modes are indicated by A, B, C and D. A, typical 1:1 interaction at DnaA box 1 with ATP- and ADP-DnaA protein; B, almost no binding to DnaA box 2 alone; C, weak cooperative binding of ADP-DnaA protein to DnaA boxes 1 and 2 in an oligonucleotide including two DnaA boxes in close distance and ATP-DnaA box a; D, cooperative binding of ATP-DnaA protein to this oligonucleotide with an ATP-DnaA box and two DnaA boxes. The lengths and relative positions of oligonucleotides in the promoter region are indicated; 1 RU corresponds to a change in mass of 1 pg/mm². Within each sensorgram, individual curves were obtained with protein concentrations (bottom to top) of 0.39, 0.78, 1.6, 3.2, 6.3, 12.5, 25, 50 and 100 nM.

basic protein. Therefore, we cannot probe concentrations >100 nM. In contrast, we used 300 nM as the highest concentration for the DNase I footprints.

Fragments with different combinations of binding sites from the *dnaA* promoter region were analyzed for their binding kinetics and stoichiometry with ATP- and ADP-DnaA protein. Representative binding experiments are shown in Figure 3. The stoichiometries and binding modes are summarized in Table I. The single consensus DnaA box 1 was bound by ATP- and ADP-DnaA protein in an identical manner (Figure 3, mode A). The data fit perfectly to a 1:1 interaction. The binding constant was 1.1 nM, as determined using the BIAevaluation software. This is similar to binding constants found by bandshift experiments with DnaA boxes from *oriC* (Schaper and Messer, 1995). The neighboring DnaA box 2, which includes a mismatch, was not bound by DnaA protein. Interestingly, DnaA boxes 1 and 2 in combination gave a stoichiometry of 2.2 for ATP-DnaA and 1.4 for ADP-DnaA protein (protein molecules:DNA molecule). Since DnaA protein

Table I. Binding of ATP- and ADP-DnaA protein to oligonucleotides with different binding sites from the *dnaA* promoter region

ATP-DnaA box/DnaA box		ADP-DnaA stoichiometry/ binding mode		ATP-DnaA stoichiometry/ binding mode	
box 1	box 2	1.0	A	1.0	A
	box 1 box 2	0.1	B	0.2	B
box a	box 1	1.4	C	2.2	D
	box 1 box 2	1.1	A	1.5	D
box a	box 1 box 2	1.7	C	3.2	D
	box b box c	1.6	C	3.1	D
box b box c		0	B	0	B

Binding of ATP- and ADP-DnaA to various oligonucleotides was studied in the BIAcore. The lengths and relative positions of oligonucleotides in the promoter are indicated. The stoichiometry factor corresponds to the number of DnaA proteins per DNA molecule at a concentration of 100 nM DnaA. Classification of binding modes corresponds to Figure 3.

did not bind to the isolated DnaA box 2, we conclude that both forms of DnaA protein bind in a cooperative way to the oligonucleotide including DnaA boxes 1 and 2. However, the DNase I footprint results (Figure 1), as well as the lower stoichiometry found in BIAcore experiments, show that ADP-DnaA is less effective in cooperative binding to DnaA box 2. Moreover, we also found that the binding kinetics for the two forms of DnaA protein were different, indicating an altered complex formation in the binding reaction of the second DnaA protein. The shapes of the binding curves in this experiment are comparable to those shown in Figure 3, modes C and D.

DnaA box 1 and ATP-DnaA box a in combination gave a stoichiometry of 1.5:1 for ATP-DnaA and 1.1 for ADP-DnaA protein. The binding curve of ADP-DnaA fits well to a 1:1 interaction (Figure 3, mode A) and the K_D was the same as for DnaA box 1 alone (1.1 nM), whereas the binding curve for ATP-DnaA was more complex. This supports the DNase I footprint results: only the ATP form of DnaA protein is able to bind to ATP-DnaA boxes. Using an oligonucleotide containing ATP-DnaA box a, DnaA box 1 and DnaA box 2, we found a stoichiometry of 3.2 for ATP-DnaA protein and 1.7 for ADP-DnaA protein (Figure 3), which corresponds well to the other BIAcore results presented above. In one case, with the oligonucleotide containing DnaA box 1, DnaA box 2, ATP-DnaA box b and ATP-DnaA box c, we were not able to reach saturation of the oligonucleotide by the highest concentration of DnaA testable (100 nM). Therefore, we could not verify the occupation of four binding sites seen at higher DnaA concentrations (166 nM)

Table II. Binding of ATP- and ADP-DnaA protein to oligonucleotides with different binding sites from the *dnaA* promoter region

ATP-DnaA box/DnaA box					ADP-DnaA stoichiometry/ binding mode		ATP-DnaA stoichiometry/ binding mode	
box a	box 1	box 2	box b	box c	1.6	C	4.3	D
box a	M	box 2	box b	box c	0	B	2.6	D
box a	M	M	box b	box c	0	B	0.7	(D)

Binding was studied in the BIAcore. Binding mode and stoichiometry were determined as in Table I. M, mutant DnaA box; (D), cannot be accurately assigned.

in DNase I footprint experiments. Instead, we found a stoichiometry of 3.1 for ATP-DnaA protein and of 1.6:1 for ADP-DnaA, which makes sense in the context of the limitations explained before. A fragment containing ATP-DnaA box b and ATP-DnaA box c, but without DnaA boxes 1 and 2, did not show any binding of ATP- and ADP-DnaA protein. This indicates that ATP-DnaA boxes are not bound if separated from DnaA boxes.

Autoregulation of *dnaA* is direct

Autoregulation of *dnaA* has been assumed to be indirect because mutant *dnaA820*, carrying three point mutations in DnaA box 1 (TTATCCACA to ATATCGAGA), had little effect on the regulation of *dnaA* (Smith *et al.*, 1997). In order to define whether this lack of repression was due to the ADP-DnaA/ATP-DnaA switch, we analyzed a DNA fragment containing ATP-DnaA boxes a, b, c and DnaA boxes 1 and 2 in the BIAcore. Binding of ATP- and ADP-DnaA protein to a wild-type and a *dnaA820* DNA fragment was tested. In addition, we tested a fragment with the new allele *dnaA821* with three more mutations in DnaA box 2 (TTTCTCACA to ATTCTGAGA). For the wild-type DNA, we observed with ATP-DnaA protein cooperative binding of mode D (Figure 3) and a stoichiometry of 4.3:1. With ADP-DnaA protein, the interaction followed mode C and the stoichiometry was 1.6. The stoichiometry was comparable to a fragment that included DnaA boxes 1 and 2 only. For the mutant allele *dnaA820*, we found an even more pronounced situation. ADP-DnaA protein did not bind to this fragment at all, but ATP-DnaA bound to this fragment cooperatively (mode D in Figure 3) with a stoichiometry of 2.6:1. The additional mutations in DnaA box 2, *dnaA821*, reduced ATP-DnaA binding strongly. The stoichiometry was only 0.7:1. ADP-DnaA did not bind. The results show that ATP-DnaA binds to both wild-type and *dnaA820* promoters, and therefore that autoregulation of the *dnaA* promoter is likely to be direct. ADP-DnaA, however, can bind to a wild-type *dnaA* promoter, but not to *dnaA820*. Fragments from the double mutant *dnaA821* were ineffective in binding any form of DnaA protein. The results are summarized in Table II.

ATP-DnaA is the primary repressor for *dnaA* transcription

The observation that ATP-DnaA binds to the *dnaA* promoter region with a higher stoichiometry than ADP-DnaA suggests that ATP-DnaA results in stronger repression. In

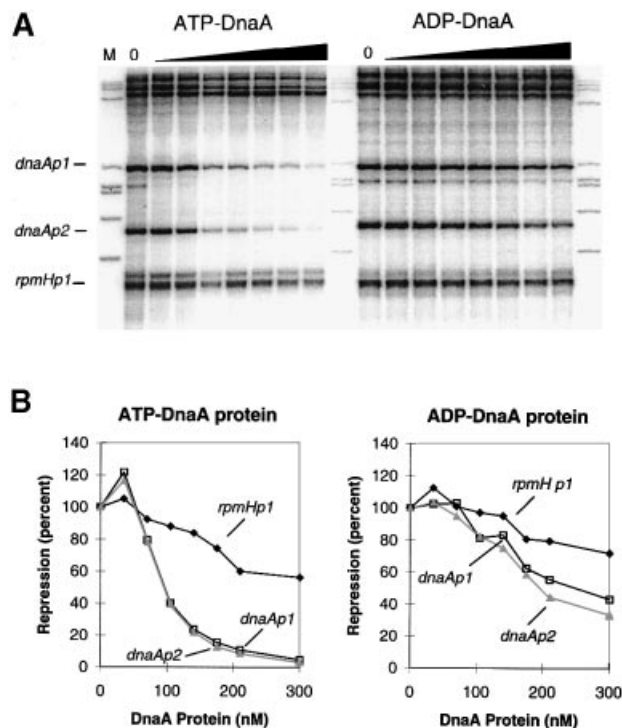


Fig. 4. ATP-DnaA protein represses the *dnaA* promoter more effectively than ADP-DnaA protein. Run-off *in vitro* transcripts from a 621 bp *EcoRI-EcoRV* restriction fragment that contains promoters *dnaAp1*, *dnaAp2* and *rpmHp1* as a control. Purified ATP- or ADP-DnaA protein (35, 70, 105, 140, 175, 210 and 300 nM) was pre-incubated with the DNA for 5 min, then RNA synthesis was started by addition of RNA polymerase (66 nM) and NTPs. The transcripts of *dnaAp1*, *dnaAp2* and *rpmHp1* are indicated (295, 212 and 178 bp, respectively). M, marker ϕ X174 *HaeIII* digest (from the bottom: 194, 234, 271, 281 and 310 bp). (A) Image of the polyacrylamide gel using a phosphorimager. (B) Quantification of the bands in (A) using ImageQuant.

order to test this, we measured the effects of ATP- and ADP-DnaA protein on the transcription of the *dnaA* gene. A 621 bp *EcoRI-EcoRV* restriction fragment contained both *dnaA* promoters and as a control the promoter of the divergently transcribed *rpmH* gene. *In vitro* run-off transcription from this fragment by RNA polymerase generated three transcripts: a transcript 295 bp in length, transcribed from the promoter *dnaAp1*; a transcript of 212 bp transcribed from *dnaAp2*; and a 178 bp fragment transcribed from *rpmHp1* (Figure 4A). ATP-DnaA protein repressed transcription from both *dnaA* promoters, *dnaAp1* and *p2*, very efficiently. Higher concentrations of ATP-DnaA resulted in nearly complete repression. In contrast, ADP-DnaA protein repressed both promoters rather weakly, and *dnaAp2* better than *dnaAp1*, but even *dnaAp2* still showed 35% activity at a concentration of 300 nM DnaA (Figure 4B). The control, the *rpmHp1* promoter, was very weakly affected by both ATP- and ADP-DnaA protein, presumably by non-specific binding. This result indicates that the ATP form of DnaA protein is the primary repressor for transcription of the *dnaA* gene.

Discussion

The promoter region of the *E.coli dnaA* gene is a complex regulatory region containing two promoters, *dnaAp1* and

dnaAp2, and two DnaA boxes located between them, a 'strong' one and a 'weak' one (Figure 2), as well as binding sites for additional factors like FIS (Froelich *et al.*, 1996) and IciA (Lee *et al.*, 1996). Using this region for an analysis of the interactions with the two forms of the DnaA protein, ATP-DnaA and ADP-DnaA, we found that: (i) both forms bind cooperatively to the two closely spaced 9mer DnaA boxes; (ii) besides binding to 9mer DnaA boxes, ATP-DnaA, but not ADP-DnaA, binds to 6mer ATP-DnaA boxes; these have a consensus sequence that is different from the 9mer DnaA box; (iii) only ATP-DnaA is an efficient repressor for the *dnaA* promoter.

DnaA protein binds cooperatively to closely spaced DnaA boxes

DnaA protein binds to individual DnaA boxes as a monomer (Schaper and Messer, 1995). DnaA boxes 1 and 2 are separated by 12 bp (Figure 2). DnaA box 1 corresponds to the stringent consensus sequence TT^A_TTNCACA. The equilibrium dissociation constant is 1.1 nM, close to the value measured for other high-affinity DnaA boxes (Schaper and Messer, 1995). DnaA box 2 has one mismatch from the consensus. Using the BIAcore, we could not detect binding to this DnaA box when it is located individually on a 21 bp double-stranded (ds) oligonucleotide. In combination with DnaA box 1, we measured a stoichiometry >1 upon binding of DnaA, for ATP- and ADP-DnaA. Binding to DnaA box 2 was also detected in DNase I footprints. This is a clear indication of cooperative binding, likely to be due to protein-protein interaction. ATP-DnaA protein bound better to DnaA box 2 than ADP-DnaA protein, seen as protection at lower DnaA concentration in the footprints (Figure 1), as well as a slower dissociation rate (Figure 3) and a higher stoichiometry in the BIAcore (Table I). This difference is due to an ATP-DnaA box that overlaps DnaA box 2 (TTTCTCACAGATTT; see below).

ATP-DnaA binds to a novel 6 bp sequence

ATP-DnaA protected sequences in DNase I footprints that were not protected by ADP-DnaA and showed no similarity to DnaA boxes (Figures 1 and 2). The stoichiometry of ATP-DnaA with fragments that contained these sequences was higher (Table I). These sequences share the consensus sequence AGATCT or a close match to it, and we call this consensus the ATP-DnaA box. Apparently, ATP-DnaA boxes are 'weak' binding sites with low affinity. Therefore, DnaA protein bound to a DnaA box is used as an anchor for binding of a second ATP-DnaA protein to an ATP-DnaA box close by, noticeable as a slow dissociation rate in the BIAcore. This results in more complex binding kinetics in the BIAcore. Thus, we assume that protein-protein interactions are stabilizing DnaA protein on the ATP-DnaA box. The BIAcore experiments show that, for example, ATP-DnaA boxes b and c are only bound by DnaA in the context of DnaA boxes 1 and 2 (Table I) or DnaA box 2 (Table II).

We conclude that both ATP- and ADP-DnaA proteins are able to interact and thus also bind cooperatively to weak DnaA boxes, but only ATP-DnaA can adopt a conformation that is able to recognize ATP-DnaA boxes. Binding of DnaA to non-canonical 5 bp sequences AGT^T/_C/A was first observed at the promoter of *oop*

Table III. ATP-DnaA boxes and the corresponding sites in λ DNA

ATP-DnaA box a	AGAACT
DnaA box 2/ATP-DnaA box	AGATTT
ATP-DnaA box b	AGATCT
ATP-DnaA box c	AGTTTA
λ 1	AGAACT
λ 2	AGATCC
λ 3	AGTCAT
Consensus	AGatct

ATP-DnaA boxes from the *dnaA* promoter region and non-canonical DnaA binding sites from λ (Szalewska-Palasz *et al.*, 1998) as defined by DNase I footprinting.

RNA in λ (Szalewska-Palasz *et al.*, 1998). Table III lists ATP-DnaA boxes identified in the *dnaA* promoter region, as well as the novel sequences found in phage λ DNA together with the consensus sequence of ATP-DnaA boxes.

The regulation of *dnaA* transcription depends on ATP-DnaA protein

It has been observed *in vivo* and *in vitro* that the *dnaA* gene is autoregulated (Atlung *et al.*, 1985; Braun *et al.*, 1985; Kücherer *et al.*, 1986; Wang and Kaguni, 1987; Polaczek and Wright, 1990; Lee and Hwang, 1997). *In vivo* transcription from both *dnaA* promoters can be repressed to 25% of normal expression by overproduction of DnaA and derepressed 3- to 4-fold by DnaA inactivation. It has been suggested that this limited regulation is instrumental in the function of DnaA as a regulator of replication initiation (Herrick *et al.*, 1996). However, a mutation that led to scrambling of DnaA box 1, *dnaA820* with three point mutations, still showed normal *in vivo* control of *dnaA* (Smith *et al.*, 1997).

In our analysis of the repression of *dnaA* transcription by ATP-DnaA and ADP-DnaA, we found that ADP-DnaA was a poor repressor *in vitro*. The 40% residual activity (Figure 4B) is apparently due to binding of ADP-DnaA to the two 9mer DnaA boxes. This shows that if cellular concentrations of ADP-DnaA are high enough, partial repression of *dnaA* is possible. ATP-DnaA, on the other hand, resulted in nearly complete repression of the *dnaA* promoter at a concentration of 300 nM, which is close to the *in vivo* concentration of DnaA (Hansen *et al.*, 1991a).

A promoter fragment with the *dnaA820* mutation bound ATP-DnaA at least 60% as efficiently as the wild-type promoter (Table II). For technical reasons, we could not reach protein saturation with the *dnaA820* fragment; therefore the DnaA-promoter stoichiometry that can be reached *in vivo* may even be higher than the 2.6 measured, and is in any case sufficient for repression (Table II). The fragment with the double-mutant *dnaA821* showed almost no binding by ATP-DnaA, and ADP-DnaA was completely inactive with both mutants.

This shows again that for binding of DnaA to weak binding sites, two boxes are required (see above and Konopa *et al.*, 1999; Majka *et al.*, 1999). In the case of ATP-DnaA, these are the weak 9mer DnaA box left intact in *dnaA820* and the adjacent ATP-DnaA boxes. For ADP-DnaA, the ATP-DnaA boxes are not accessible and therefore binding is not possible. The results on DnaA

binding and transcription *in vitro* easily explain the *in vivo* results obtained with the *dnaA820* mutant (Smith *et al.*, 1997). We conclude that ATP-DnaA is the principal repressor for the *dnaA* gene, and that autoregulation is direct.

A comparison of the promoter sequences of other genes that are controlled by DnaA protein suggests that ATP-DnaA may play a similar role in their regulation. We found sequences conforming to ATP-DnaA boxes in the promoter regions of various genes, e.g. *rpoH*, *mioC*, *mutH*, *polA* and the *nrd* operon, suggesting that these genes are also regulated in the context of DNA replication. However, experimental proof is still lacking.

The ATP/ADP-DnaA switch in the cell cycle

ATP-DnaA is required for replication initiation since only the ATP form promotes local unwinding at the AT-rich region of *oriC* (Bramhill and Kornberg, 1988; Sekimizu *et al.*, 1988). Interestingly, unwinding requires some but not all DnaA in the ATP form (Yung *et al.*, 1990). During initiation, DnaA also binds to the AT-rich region, as inferred from competition filter-binding experiments (Yung and Kornberg, 1989). Each of the AT-rich 13mers in *oriC* starts with an ATP-DnaA box consensus sequence. We speculate that the function of DnaA in unwinding is exerted by binding to these ATP-DnaA boxes. This question is currently under investigation. In the last step of the initiation process, the inherent ATPase of DnaA is activated by loading of the β subunit of DNA polymerase III holoenzyme, the sliding clamp, and subsequent DNA synthesis, and thereby DnaA is inactivated for further initiation events (Katayama *et al.*, 1998).

Local unwinding at a replication origin is one of the basic processes that occurs at the origins of most organisms. The biochemistry of this process is not known in any system. As an example, the eukaryotic origin recognition complex (ORC) in *Saccharomyces cerevisiae* is a six protein complex with a function analogous to DnaA protein in bacteria. Origin recognition by ORC requires ATP, and one of its subunits hydrolyzes ATP (Klemm *et al.*, 1997). Whether there is also a function for the ADP-ORC complex is at present unknown.

Newly produced DnaA protein is likely to be in the ATP form, since ATP is much more abundant in the cell. During replication, ATP-DnaA protein is hydrolyzed; 80% of the DnaA protein was found to be in the ADP form (Katayama *et al.*, 1998). Therefore, during active replication, little repression of *dnaA* expression occurs and predominantly ADP-DnaA accumulates to bind to the increasing number of DnaA boxes around the chromosome. At *oriC*, ADP-DnaA may build up the larger part of the initial initiation complex. Only a minority of the DnaA protein will be in the ATP form, but this is the fraction that provides the critical molecules triggering the initiation process. In the absence of replication, ATP-DnaA protein is stable and accumulates in the cell, leading to a rapid cessation of *dnaA* transcription (Figure 5).

Measurements of cellular DnaA concentrations at different growth rates and upon different treatments, and models derived from these, have been based so far on total DnaA protein (sum of ATP- and ADP-DnaA) (Hansen *et al.*, 1991a,b; see also Herrick *et al.*, 1996; Messer and Weigel, 1996 and citations therein). In view of our results, we

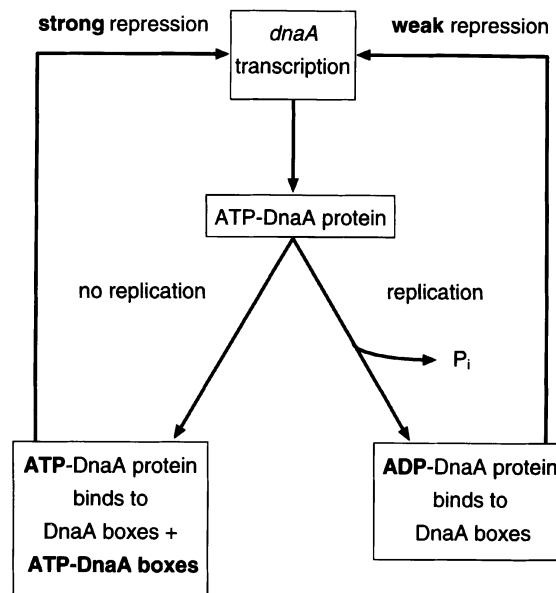


Fig. 5. Autoregulation of the *dnaA* gene by ATP- and ADP-DnaA protein. A model for *in vivo* regulation of DnaA protein synthesis. Newly synthesized DnaA protein will be in the ATP form. During exponential growth of *E. coli*, DNA replication is rapid, ATP-DnaA will be hydrolyzed to ADP-DnaA in a Pol III β -subunit-dependent manner (Katayama *et al.*, 1998). The larger fraction of DnaA protein in the cell will then be in the ADP form and will be titrated by DnaA boxes. Binding to DnaA boxes causes only weak repression of *dnaA* transcription. If replication is blocked, e.g. in stationary phase, ATP-DnaA protein is stable in the cell and can bind to ATP-DnaA boxes at the *dnaA* promoter, strongly repressing *dnaA* transcription.

need now to include measurements of the different forms of DnaA, as has been suggested in one of the models of initiation regulation (Mahaffy and Zyskind, 1989).

Molecular switches that depend on nucleotide binding and hydrolysis for the definition of an on or off status have been found in many systems. These include GTP-dependent switches in G proteins involved in signal transduction (for a review, see Sprang, 1997) and ATP-regulated switches in transposition (Yamauchi and Baker, 1998) and mismatch repair (Gradia *et al.*, 1997). To our knowledge, only two examples are known of a switch that allows discrimination between two alternative pathways rather than progression through one pathway. One is the partition system of plasmid P1 where protein-protein interaction of ParA and ParB, and thus the activity of the partition complex, depends on ATP-ParA. Autoregulation due to binding of ParA to the operator of the *par* operon is, however, dependent on ADP-ParA (Bouet and Funnell, 1999).

The second example is the system described here. An ATP/ADP switch for DnaA as a regulatory protein defines alternative DNA-binding specificity. ATP-DnaA serves as a repressor and provides the few ATP-DnaA molecules that are required for local DNA unwinding in the critical step of the replication initiation process. ADP-DnaA binds to the many DnaA boxes that are located around the chromosome, and in this way neutralizes their capacity to titrate away ATP-DnaA. Within *oriC*, ADP-DnaA is likely to build up the larger part of the initiation complex, and thereby forms the core of the specialized nucleoprotein structure at *oriC* (Echols, 1990).

Table IV. Oligonucleotides

Footprint oligonucleotides	
pdnaA footprint 5'up	5'-AATTTTCCAATATGCCGCGTAAATCGTGC-3'
pdnaA footprint 3'lo	5'-GAACCGCTGTCTGCGGTTATATGC-3'
BIAcore oligonucleotides	
pdnaA 1	5'-Bio-CCCGATTATCCACAGGACTT-3'
pdnaA 2	5'-Bio-GATCTTCTGTTTCTACAGATTTTCCC-3'
pdnaA 1,2	5'-Bio-CCCGATTATCCACAGGACTTTCCAGAACTC GCT-3'
pdnaA a,1	5'-Bio-CCCGATTATCCACAGGACTTTCCAGAACTC GCTAAG-3'
pdnaA a,1,2	5'-Bio-GATCTTCTGTTTCTACAGATTTTCCCGATTATCCACAGGACTTTCCAGAACTCGCTAAG-3'
pdnaA 1,2,b,c	5'-Bio-CCGCGGATCATAGCCTAAACTGCGCAAGAGATCTTCTGTTTCTACAGATTTTCCCGATTATCCA CAGGACTT-3'
pdnaA b,c	5'-Bio-CCGCGGATCATAGCCTAAACTGCGCAAGAGATCTTCTGTTTCTGTT-3'
pdnaA a,1,2,b,c	5'-Bio-CCGCGGATCATAGCCTAAACTGCGCAAGAGATCTTCTGTTTCTACAGATTTTCCCGATTATCCA CAGGACTTTCCAGAACTCGCTAAG-3'
pdnaA820	5'-Bio-CCGCGGATCATAGCCTAAACTGCGCAAGAGATCTTCTGTTTCTACAGATTTTCCCGATATATCGA GAGGACTTTCCAGAACTCGCTAAG-3'
pdnaA821	5'-Bio-CCGCGGATCATAGCCTAAACTGCGCAAGAGATCTTCTGATTCTGAGAGATTTTCCCGATATATCGA GAGGACTTTCCAGAACTCGCTAAG-3'

The footprint oligonucleotides were utilized to generate a 198 bp PCR fragment, containing the dnaA promoter region. For the BIAcore oligonucleotides only the lower strand is shown. The complementary strand was without biotin marker.

Materials and methods

Chemicals, proteins and oligonucleotides

Standard chemicals were obtained from Sigma (St Louis, MO) or Merck (Darmstadt, Germany) and radioactive [γ - 32 P]ATP (3000 Ci/mmol) from Amersham (Little Chalfont, UK). Restriction enzymes were supplied by Boehringer Mannheim (Germany) or New England Biolabs (Beverly, MA). *Escherichia coli* RNA polymerase was from Boehringer Mannheim (Germany) and dialysis membranes from Millipore (Bedford, MA). Oligonucleotides used for PCR and BIAcore were synthesized chemically (Gibco-BRL, Gaithersburg, MD).

DnaA protein was overexpressed from plasmid pdnaA116 and purified as described (Krause *et al.*, 1997). Standard methods were used for DNA manipulations (Sambrook *et al.*, 1989).

Dialysis

Because of the slow rate of ADP-ATP exchange in DnaA, we dialyzed the protein extensively against ATP or ADP. Saturation of the exchange was monitored using the difference of the two forms in the BIAcore; 100 μ l of DnaA protein (850 μ g/ml) were incubated for 24 h with 3 mM ATP or ADP. We then dialyzed for 2 h against 20 ml of G buffer [45 mM HEPES-KOH pH 7.6, 750 mM potassium glutamate, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 20% sucrose, 3 mM ATP or ADP] using a 0.025 μ m MF-Millipore membrane. All steps for dialysis were performed at 4°C. After dialysis, the protein was kept at 4°C for 24 h before being used in experiments.

DNase I protection assays

DNase I protection assays were performed as described (Galas and Schmitz, 1978) with minor modifications. DNA fragments were amplified using the primers shown in Table IV, one primer 5' end-labeled with 32 P and T4 polynucleotide kinase. Binding reactions were carried out with 5 ng of labeled PCR fragments in 20 μ l of the binding buffer [25 mM HEPES pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 5 mM calcium acetate, 4 mM DTT, 0.2% Triton X-100, 0.5 mg/ml bovine serum albumin (BSA), 3 mM ATP or ADP] and the indicated amounts of ATP- or ADP-DnaA. Mixtures were incubated at 37°C for 10 min, DNase I (5 pg) was added and samples were incubated at 37°C for 4 min. After addition of an equal volume of stop buffer (1% SDS, 200 mM NaCl, 20 mM EDTA pH 8.0, 1 mg/ml glycogen), the samples were purified by phenol-chloroform extraction, DNA was precipitated with ethanol and resuspended in 1 μ l of TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). Following addition of 5 μ l of sequencing gel loading buffer (98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol), samples were incubated at 96°C for 5 min and loaded onto 8% sequencing gels. After electrophoresis, gels were dried and autoradiographed.

Surface plasmon resonance

Surface plasmon resonance was determined using a BIAcore 2000 instrument (BIAcore AB, Uppsala, Sweden). To exclude non-specific

binding, we used a reference flow cell with non-specific DNA and included competitor [poly(dI-dC) + poly(dA-dT)] in the binding buffer. Mass transfer effects could be excluded using a high flow rate of 100 μ l/min and a low amount of DNA (<50 RU) coupled to the sensor chip surface. The streptavidin-coated Sensor Chip SA was conditioned as recommended by the manufacturer and in addition washed with 0.05% SDS in TES (10 mM Tris pH 8.0, 300 mM NaCl, 1 mM EDTA) for 3 min in short pulses. Biotinylated ds oligonucleotides diluted in TES were immobilized using TES as the flow buffer. The DnaA protein was diluted in HKM buffer (25 mM HEPES pH 7.6, 100 mM potassium acetate, 1 mM magnesium acetate, 0.005% BIAcore Surfactant P20) and poly(dI-dC) and poly(dA-dT) were added as a competitor at a final concentration of 33 ng/ μ l each. Samples in a concentration range of 0.39–100 nM DnaA were injected using the kinject command defining an association time of 220 s and a dissociation time of 300 s. Reactions were performed at 22°C except for the experiments in Table II, which were performed at 37°C. At the end of each cycle, bound DnaA was removed with a 5 μ l pulse of 0.005% SDS in TES. For data analysis, we used the BIAevaluation 3.0 program (BIAcore AB, Sweden, Uppsala).

Stoichiometry

Resonance response for ds DNA in comparison with protein was determined similarly to the procedure for single-stranded DNA (Fisher *et al.*, 1994). An average from two measurements each on five different oligonucleotides with single binding sites gave:

$$RU_{\text{protein}} = 0.73 RU_{\text{ds DNA}}$$

Stoichiometry was determined at a concentration of 100 nM DnaA protein, approaching saturation for complex formation.

In vitro transcription

The *in vitro* transcription assay was performed as described (Escobar *et al.*, 1997) with minor modifications. A 621 bp *EcoRI*–*EcoRV* restriction fragment from plasmid pLSK4 that contains the *dnaA* promoter region was used as a linear template for single-round run-off transcription assays. These were carried out at 37°C in transcription buffer (25 mM HEPES pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 4 mM DTT, 0.2% Triton X-100, 0.5 mg/ml BSA, 3 mM ATP or ADP) in a total volume of 20 μ l. The DNA template (10 ng) was pre-incubated for 5 min with variable amounts of DnaA protein as indicated in the figure legends. Subsequently, RNA polymerase was added to a concentration of 66 nM, followed by another 5 min of incubation. The transcription reactions were initiated by addition of [α - 32 P]UTP (7.5 μ Ci, 3000 Ci/mmol), unlabeled UTP (60 μ M), ATP (200 μ M), CTP and GTP (each at 400 μ M), and heparin (200 μ g/ml). Mixtures were incubated for 5 min and then the reaction was terminated by adding stop buffer. Purification and electrophoresis were performed as for DNase I footprints.

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References

- Atlung,T., Clausen,E. and Hansen,F.G. (1985) Autoregulation of the *dnaA* gene of *Escherichia coli*. *Mol. Gen. Genet.*, **200**, 442–450.
- Bouet,J.-Y. and Funnell,B.E. (1999) P1 ParA interacts with the P1 partition complex at *parS* and an ATP–ADP switch controls ParA activities. *EMBO J.*, **18**, 1415–1424.
- Bramhill,D. and Kornberg,A. (1988) Duplex opening by *dnaA* protein at novel sequences in initiation of replication at the origin of the *E.coli* chromosome. *Cell*, **52**, 743–755.
- Braun,R.E., O'Day,K. and Wright,A. (1985) Autoregulation of the DNA replication gene *dnaA* in *E.coli*. *Cell*, **40**, 159–169.
- Castuma,C.E., Crooke,E. and Kornberg,A. (1993) Fluid membranes with acidic domains activate DnaA, the initiator protein of replication in *Escherichia coli*. *J. Biol. Chem.*, **268**, 24665–24668.
- Echols,H. (1990) Nucleoprotein structures initiating DNA replication, transcription, and site-specific recombination. *J. Biol. Chem.*, **265**, 14697–14700.
- Escolar,L., De Lorenzo,V. and Perez-Martin,J. (1997) Metalloregulation *in vitro* of the aerobactin promoter of *Escherichia coli* by the Fur (ferric uptake regulation) protein. *Mol. Microbiol.*, **26**, 799–808.
- Fisher,R.J., Fivash,M., Casas-Finet,J., Bladen,S. and Larsen McNitt,K. (1994) Real-time BIAcore measurements of *Escherichia coli* single-stranded DNA binding (SSB) protein to polydeoxythymidylic acid reveal single-state kinetics with steric cooperativity. *Methods Enzymol.*, **6**, 121–133.
- Froelich,J.M., Phuong,T.K. and Zyskind,J.W. (1996) Fis binding in the *dnaA* operon promoter region. *J. Bacteriol.*, **178**, 6006–6012.
- Galas,D. and Schmitz,A. (1978) Dnase footprinting: a simple method for determining protein–DNA binding specificity. *Nucleic Acids Res.*, **5**, 3157–3170.
- Gradia,S., Acharya,S. and Fishel,R. (1997) The human mismatch recognition complex hMSH2–hMSH6 functions as a novel molecular switch. *Cell*, **91**, 995–1005.
- Hansen,E.B., Hansen,F.G. and von Meyenburg,K. (1982) The nucleotide sequence of the *dnaA* gene and the first part of the *dnaN* gene of *Escherichia coli* K12. *Nucleic Acids Res.*, **10**, 7373–7385.
- Hansen,F.G., Atlung,T., Braun,R.E., Wright,A., Hughes,P. and Kohiyama,M. (1991a) Initiator (DnaA) protein concentration as a function of growth rate in *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.*, **173**, 5194–5199.
- Hansen,F.G., Christensen,B.B. and Atlung,T. (1991b) The initiator titration model: computer simulation of chromosome and minichromosome control. *Res. Microbiol.*, **142**, 161–167.
- Herrick,J., Kohiyama,M., Atlung,T. and Hansen,F.G. (1996) The initiation mess? *Mol. Microbiol.*, **19**, 659–666.
- Kaguni,J.M. (1997) *Escherichia coli* DnaA protein: the replication initiator. *Mol. Cells*, **7**, 145–157.
- Katayama,T., Kubota,T., Kurokawa,K., Crooke,E. and Sekimizu,K. (1998) The initiator function of DnaA protein is negatively regulated by the sliding clamp of the *E.coli* chromosomal replicase. *Cell*, **94**, 61–71.
- Klemm,R.D., Austin,R.J. and Bell,S.P. (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. *Cell*, **88**, 493–502.
- Konopa,G., Szalewska-Palasz,A., Schmidt,A., Srutkowska,S., Messer,W. and Wegrzyn,G. (1999) The presence of two DnaA-binding sequences is required for efficient interaction of the *Escherichia coli* DnaA protein with each particular weak DnaA box region. *FEMS Microbiol. Lett.*, **174**, 25–31.
- Kornberg,A. and Baker,T.A. (1992) *DNA Replication*. W.H.Freeman and Co., New York, NY.
- Krause,M., Lurz,R., Rückert,B. and Messer,W. (1997) Complexes at the replication origin of *Bacillus subtilis* with homologous and heterologous DnaA protein. *J. Mol. Biol.*, **274**, 365–380.
- Kücherer,C., Lother,H., Kölling,R., Schauzu,M.A. and Messer,W. (1986) Regulation of transcription of the chromosomal *dnaA* gene of *Escherichia coli*. *Mol. Gen. Genet.*, **205**, 115–121.
- Lee,Y.S. and Hwang,D.S. (1997) Occlusion of RNA polymerase by oligomerization of DnaA protein over the *dnaA* promoter of *Escherichia coli*. *J. Biol. Chem.*, **272**, 83–88.
- Lee,Y.S., Kim,H. and Hwang,D.S. (1996) Transcriptional activation of the *dnaA* gene encoding the initiator for *oriC* replication by IciA protein, an inhibitor of *in vitro oriC* replication in *Escherichia coli*. *Mol. Microbiol.*, **19**, 389–396.
- Mahaffy,J.M. and Zyskind,J.W. (1989) A model for the initiation of replication in *Escherichia coli*. *J. Theor. Biol.*, **140**, 453–477.
- Majka,J., Jakimowicz,D., Messer,W., Schrepf,H., Lisowski,M. and Zakrzewska-Czerwinska,J. (1999) Interactions of the *Streptomyces lividans* initiator protein DnaA with its target. *Eur. J. Biochem.*, **260**, 325–335.
- Messer,W. and Weigel,C. (1996) Initiation of chromosome replication. In Neidhardt,F.C. *et al.* (eds), *Escherichia coli and Salmonella, Cellular and Molecular Biology*. ASM, Washington, DC, pp. 1579–1601.
- Messer,W. and Weigel,C. (1997) DnaA initiator—also a transcription factor. *Mol. Microbiol.*, **24**, 1–6.
- Mizushima,T., Nishida,S., Kurokawa,K., Katayama,T., Miki,T. and Sekimizu,K. (1997) Negative control of DNA replication by hydrolysis of ATP bound to DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*. *EMBO J.*, **16**, 3724–3730.
- Polaczek,P. and Wright,A. (1990) Regulation of expression of the *dnaA* gene in *Escherichia coli*: role of the two promoters and the DnaA box. *New Biol.*, **2**, 574–582.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schaper,S. and Messer,W. (1995) Interaction of the initiator protein DnaA of *Escherichia coli* with its DNA target. *J. Biol. Chem.*, **270**, 17622–17626.
- Sekimizu,K., Bramhill,D. and Kornberg,A. (1987) ATP activates *dnaA* protein in initiating replication of plasmids bearing the origin of the *E.coli* chromosome. *Cell*, **50**, 259–265.
- Sekimizu,K., Bramhill,D. and Kornberg,A. (1988) Sequential early stages in the *in vitro* initiation of replication at the origin of the *Escherichia coli* chromosome. *J. Biol. Chem.*, **263**, 7124–7130.
- Skarstad,K. and Boye,E. (1994) The initiator protein DnaA: evolution, properties and function. *Biochim. Biophys. Acta*, **1217**, 111–130.
- Smith,R.W.P., McAteer,S. and Masters,M. (1997) Autoregulation of the *Escherichia coli* replication initiator protein, DnaA, is indirect. *Mol. Microbiol.*, **23**, 1303–1315.
- Sprang,S.R. (1997) G protein mechanisms: insights from structural analysis. *Annu. Rev. Biochem.*, **66**, 639–678.
- Szalewska-Palasz,A. *et al.* (1998) Interaction of the *Escherichia coli* DnaA protein with λ phage DNA. *Mol. Gen. Genet.*, **259**, 679–688.
- Wang,Q. and Kaguni,J.M. (1987) Transcriptional repression of the *dnaA* gene of *Escherichia coli* by *dnaA* protein. *Mol. Gen. Genet.*, **209**, 518–525.
- Yamauchi,M. and Baker,T.A. (1998) An ATP–ADP switch in MuB controls progression of the Mu transposition pathway. *EMBO J.*, **17**, 5509–5518.
- Yung,B.Y.M. and Kornberg,A. (1989) The *dnaA* initiator protein binds separate domains in the replication origin of *Escherichia coli*. *J. Biol. Chem.*, **264**, 6146–6150.
- Yung,B.Y.M., Crooke,E. and Kornberg,A. (1990) Fate of the DnaA initiator protein in replication at the origin of the *Escherichia coli* chromosome *in vitro*. *J. Biol. Chem.*, **265**, 1282–1285.

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